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Urine CXCL1 as a biomarker for tumor detection and outcome prediction in bladder cancer

Masakazu Nakashima[a], Yoshiyuki Matsui[a], Takashi Kobayashi[a], Ryoichi Saito[a], Satoko Hatahira[b], Kazumi Kawakami[b], Eijiro Nakamura[c], Hiroyuki Nishiyama[d] and Osamu Ogawa[a,*]
[a]*Department of Urology, Kyoto University Graduate School of Medicine, Kyoto, Japan*
[b]*Toray Industries Inc., New Frontiers Research Laboratories, Kanagawa, Japan*
[c]*Laboratory for Malignancy Control Research, Medical Innovation Center, Kyoto University Graduate School of Medicine, Kyoto, Japan*
[d]*Department of Urology, Faculty of Medicine, University of Tsukuba, Tsukuba, Japan*

Abstract.
BACKGROUND AND OBJECTIVE: To clarify the clinical usefulness and diagnostic accuracy of urine chemokine (C-X-C motif) ligand 1 (CXCL1) as a biomarker for tumor detection and outcome prediction in patients with bladder cancer (BCa).
METHODS: We measured urine CXCL1 levels in 175 patients with BCa and 30 healthy controls. The value of urine CXCL1 concentration normalized by urine creatinine (CXCL1/Cre) was analyzed in terms of detecting bladder tumors and predicting intravesical recurrence after transurethral resection (TUR).
RESULTS: CXCL1/Cre was significantly higher (3-fold) in BCa patients than in healthy participants and the difference from control samples was greater in patients with advanced BCa. Although the urine cytology test generally lost diagnostic power in patients with low-grade superficial tumors, the sensitivity of CXCL1/Cre was not compromised in this patient population. Patients with higher CXCL1/Cre were significantly more likely to develop intravesical recurrence after TUR and multivariate analysis identified CXCL1/Cre as an independent predictor of post-TUR intravesical recurrence. Importantly, CXCL1/Cre could successfully classify the probabilities of post-TUR recurrence among patients with intermediate-risk according to EORTC risk criteria into two groups equivalent to its high- and low-risk groups.
CONCLUSIONS: Urine CXCL1 is a promising, non-invasive molecular marker for tumor detection and outcome prediction in patients with BCa.

Keywords: Bladder cancer, urine biomarker, sensitivity and specificity, detection, prognostic factor

1. Introduction

Bladder cancer (BCa) is the sixth most common cancer worldwide [1,2]. Approximately 70% of BCas do not invade the muscle, meaning that the majority of cases can be resected transurethrally with excellent survival outcomes. However, repeated intravesical recurrence is the main problem in noninvasive BCa, given that the cost of observation is higher than for other cancers [3]. Of patients with non-muscle-invasive BCa, up to 80% will have recurrent disease and 45% will experience disease progression [4].

Although urine cytology has been broadly used as noninvasive clinical test for detection of BCa, it has low sensitivity for the detection of low-grade BCa. Cystoscopic examination of the bladder thus remains the gold standard. Various molecular markers have been studied to date, but the current European Association of Urology and American Urological Association

*Corresponding author: Osamu Ogawa, Department of Urology, Kyoto University Graduate School of Medicine, 54 Shogoin-kawahara-cho, Sakyo-ku, Kyoto 606-8507, Japan. Tel.: +81 75 751 3325; Fax: +81 75 761 3441; E-mail: ogawao@kuhp.kyoto-u.ac.jp.

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Table 1
Clinicopathologic characteristics of patients with bladder cancer

	Overall ($n = 175$)	Primary ($n = 96$)	Recurrent ($n = 79$)
Age			
Mean	70.6	69.7	71.8
(sd)	(9.9)	(9.5)	(10.2)
Sex			
M	132 (75.4%)	74 (77.1%)	58 (73.4%)
F	43 (24.6%)	22 (22.9%)	21 (26.6%)
Smoking			
Past or ever	108 (61.7%)	63 (65.6%)	45 (57.0%)
Never	67 (38.3%)	33 (34.4%)	34 (43.0%)
Tumor pathology at TUR			
pTis	8 (4.6%)	4 (4.2%)	4 (5.1%)
pTa, LG	60 (34.3%)	23 (24.0%)	37 (46.8%)
pTa, HG	48 (27.4%)	21 (21.9%)	27 (34.2%)
pT1	36 (20.6%)	30 (31.3%)	6 (7.6%)
pT2 or more	23 (13.1%)	18 (18.8%)	5 (6.3%)
Past history of upper UC			
Yes	18 (10.3%)	0 (0%)	18 (22.8%)
No	157 (89.7%)	96 (100%)	61 (77.2%)

TUR, transurethral resection; LG, low grade; HG, high grade; UC, urothelial cancer.

guidelines state that the use of urine-based markers for BCa detection should not be strongly recommended because of their generally low specificity [5,6].

The medical and economic burdens associated with BCa follow-up may also be reduced by prioritizing screening for high-risk patients, by optimization and individualization of treatment selection and follow-up period. In this regard, the European Organisation for Research and Treatment of Cancer (EORTC) risk-scoring system stratifies patients into low-, intermediate- and high-risks based on six clinical and pathological factors: number of tumors, tumor size, prior recurrence rate, T category, carcinoma in situ, and grade. Based on the risk stratification, the estimated probabilities of recurrence at 1 year are 15%, 24–38% and 61% for patients with low-, intermediate- and high-risk BCa, respectively. However, a limitation of this approach is that approximately 80% of patients are classified in the intermediate-risk group [7]. Further, the EORTC criteria system encounters another problem when deciding on the application of immediate intravesical instillation after transurethral resection (TUR); decision-making at TUR based on EORTC risk criteria is difficult, because pathological factors are necessary for the classification.

A molecular marker able to predict the presence of tumor and risk of post-TUR recurrence is therefore needed. We previously reported that secreted protein chemokine (C-X-C motif) ligand 1 (CXCL1) was upregulated in highly-invasive BCa cell lines and clinically-obtained BCa specimens, as well as in patient urine [8]. Similar results were reported by Miyake et al. in BCa specimens [9]. The ratio of urine CXCL1 to creatinine (CXCL1/Cre) was significantly higher in patients with invasive BCa (pT1-4) compared with those with noninvasive pTa tumor, and also showed significant elevation even in early-stage tumors [8]. In this study, we aimed to evaluate the diagnostic accuracy of urine CXCL1 for bladder cancer detection and clinical outcome prediction. As a result we have demonstrated the usefulness of urine CXCL1 for both the detection of BCa tumors and prediction of future recurrence, particularly in low-grade tumors. These results may improve the management of patients with superficial BCa.

2. Patients and methods

2.1. Study subjects and sample collection

Urine specimens and associated clinical information were prospectively collected from 201 consecutive patients with BCa diagnosed at Kyoto University Hospital from October 2005 to February 2010. This study on human subjects was approved by Kyoto University Hospital Institutional Review Board and by the Human Tissue Samples Ethics Committee for R&D, Toray Industries Inc. Written informed consent was obtained from each participant. Six samples from patients with concomitant acute UTIs or past urological interventions (such as radiation therapy and bladder irrigation) and 20 samples from duplicated individuals were excluded. A total of 175 samples were thus analyzed for

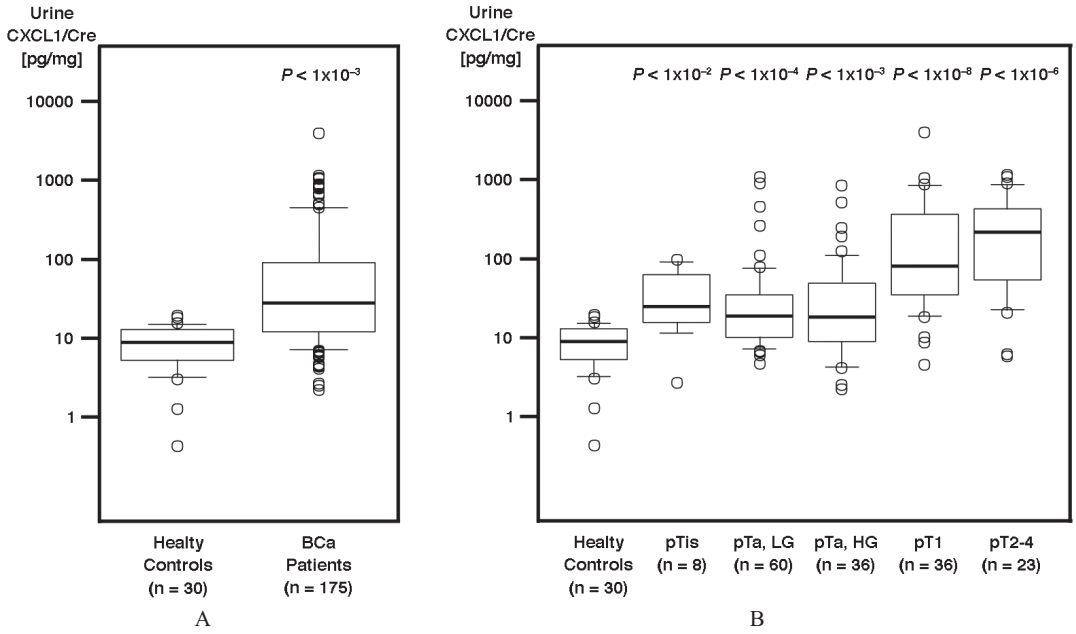

Fig. 1. Box plots of urine CXCL1/Cre. Boxes and bars represent interquartile range and 10–90 percentile range, respectively. Bars within boxes represent median values. A: Healthy controls and patients. B: Healthy controls and patients stratified according to tumor pathology at TUR. P values for pairwise comparisons between each pathological group and healthy group were adjusted by Steel's test.

urine CXCL1/Cre levels and urine cytology. All urine cytology tests were performed using fresh urine samples.

All bladder cancers were diagnosed using cystoscopic examination and pathologically confirmed using TUR specimen. Of the 175 patients, 96 were newly diagnosed and 79 had recurrent disease. The clinicopathological characteristics of the 175 patients are shown in Table 1. We also analyzed urine samples from 30 healthy controls. Median follow-up after TUR was 4.5 years. Patients who underwent complete resection by TUR with preservation of the urinary bladder were followed up with cystoscopy and urine cytology every 3 months postoperatively.

2.2. Measurement of urine CXCL1 levels

All urine samples were collected at pre-TUR settings and examined by two of the authors (S.H. and K.K.) who were blinded to clinicopathological information including the presence, pathological stage, histological grade of the bladder tumor.

The samples were centrifuged for 10 min at 2,000 rpm at room temperature to remove debris, aliquoted, and stored at -80°C. On the day of analysis, frozen urine samples were thawed rapidly and urinary CXCL1 levels were measured using a sandwich ELISA system (see Supplementary Material for details) newly developed by Toray Industries Inc (Tokyo, Japan), with original monoclonal antibodies. Urine creatinine levels were measured spectrophotometrically using the alkaline picrate method.

2.3. Statistical analysis

All results were entered into a database for statistical analysis. Nonparametric Mann-Whitney U tests and Kruskal-Wallis tests were used to compare BCa subgroups and healthy controls. P values were adjusted by Steel's test. Urine CXCL1 levels were normalized by urine creatinine (CXCL1/Cre, pg/mg). The diagnostic ability of CXCL1/Cre for the detection of BCa was evaluated using the receiver-operating characteristic (ROC) method. χ^2 tests were performed to evaluate differences in diagnostic performance between CXCL1/Cre and urine cytology. Kaplan-Meier analysis with log-rank test and Cox's proportional hazards test were used to analyze post-TUR recurrence-free survival (RFS) in patients with pTa and pT1 BCa ($n = 142$). Statistical analysis was performed using 'EZR' (Easy R) software, which is based on R and R commander [10]. All tests were two-sided, and a P value of < 0.05 was considered statistically significant.

3. Results

3.1. Detection of BCa

Overall, CXCL1/Cre was significantly higher in BCa patients compared with healthy controls (Fig. 1A). The median CXCL1/Cre value was 3-fold higher in BCa patients than in healthy controls. The difference increased with increasing tumor stage (Fig. 1B). CXCL1/Cre ratios in pT1 and pT2-4 BCa were 10- and 32-fold higher than in controls, respectively. The same trends were observed in patients with primary and recurrent BCa (Supplementary Fig. 1).

ROC analysis on 205 subjects (175 BCa patients and 30 healthy control) for the detection of BCa exhibited an area under the curve (AUC) of 0.84 (95% confidence interval: 0.78–0.89) (Fig. 2). Importantly, the detection rate of CXCL1/Cre compared with the conventional cytology test was particularly remarkable in patients with pTa low-grade tumors (Table 2). The sensitivities of the urine cytology test were as low as 22–35% and 5–18% with cutoff values of class 3 and 4, respectively, while CXCL1/Cre achieved a sensitivity of 48–54% in the same subjects using a cutoff of value providing 95% specificity (18.3) ($P = 0.006$ and $P < 0.001$, χ^2 test with Yates' continuity correction compared with class 3 and 4 cytologies, respectively). CXCL1/Cre showed similar sensitivity with the urine cytology test in other subjects.

3.2. Prediction of post-TUR intravesical recurrence

The impact of CXCL1/Cre on prognosis was measured by analyzing intravesical RFS by Kaplan-Meier analysis. Median RFS in patients with CXCL1/Cre $\geqslant$ 35 was significantly poorer than in patients with CXCL1/Cre <35 (295 and 974 days, respectively, $P = 0.018$, log-rank test) (Fig. 3A). This difference was robust using cutoff values of 25–40 for CXCL1/Cre (data not shown). CXCL1/Cre was able to separate patients with EORTC intermediate-risk into those with good and poor RFS equivalent to EORTC low- and high-risk populations, respectively (Fig. 3B and C). Among EORTC intermediate-risk patients ($n = 120$), the median RFSs were 379 and 1104 days for patients with CXCL1/Cre $\geqslant$ 35 and $<$ 35, respectively ($P = 0.049$, log-rank test). Moreover, EORTC high-risk patients with CXCL1/Cre $\geqslant$ 35 had significantly poorer RFS ($P = 0.006$, log-rank test) (Fig. 3D).

Collectively, these results suggest that CXCL1/Cre could predict BCa tumor recurrence in both primary and recurrent tumors, and irrespective of EORTC risk classification. A Cox proportional hazards model incorporating smoking, past history of upper tract urinary cancer, urine cytology, EORTC recurrence risk and urine CXCL1/Cre revealed that CXCL1/Cre was a significant predictor of post-TUR recurrence (Table 3). Urine cytology did not predict post-TUR recurrence in any EORTC risk group in the present study.

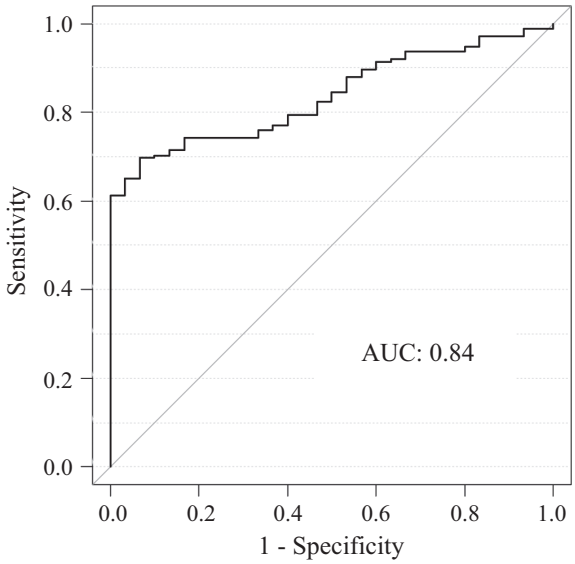

Fig. 2. Receiver operating characteristic (ROC) curve for urine CXCL1/Cre for the diagnosis of bladder cancer. ROC analysis was performed for urine CXCL1 normalized to Cre ($n = 205$; $n = 175$ patients with bladder cancer and $n = 30$ healthy controls). Area under the curve (AUC) for CXCL1/Cre is 0.84 (95% confidence interval: 0.78–0.89).

4. Discussion

The results of this study identified CXCL1 as a promising urine marker for BCa detection, particularly in low-grade tumors, and demonstrated the potential of urine CXCL1 as a prognostic marker in noninvasive BCa.

CXCL1 belongs to the CXC chemokines, which contribute to normal biological reactions such as the chemoattraction of leukocytes to inflammatory sites. Especially with regard to cancers, it is also likely to modulate tumor angiogenesis, cell proliferation and metastasis [11]. We previously reported that CXCL1 was increased in urine from BCa patients, and that urine CXCL1, as well as urine cytology, was an independent factor predicting the existence of BCa with invasive phenotype [8]. Several reports have shown

Table 2
Sensitivities of urine CXCL1/Cre and urine cytology

CXCL1/Cre	Cytology		
Cut off	18.3 (specificity = 95%)	3 or more	4 or 5
Primary ($n = 95$)	71.9%	71.0 %	54.7 %
pTis ($n = 4$)	50.0%	100%	100%
pTa, LG ($n = 23$)	47.8%	34.8%	17.4%
pTa, HG ($n = 21$) 57.1% 57.1% 28.6%			
pT1 ($n = 29$)	90.0%	96.6%	86.2%
pT2-4 ($n = 18$) 94.4% 94.4% 72.2%			
Recurrent ($n = 79$)	57.0%	38.0%	11.4%
pTis ($n = 4$)	75.0%	75.0%	25.0%
pTa, LG ($n = 37$)	54.1%	21.6%	5.4%
pTa, HG ($n = 27$)	44.4%	40.7%	7.4%
pT1 ($n = 6$)	100%	66.7%	33.3%
pT2-4 ($n = 5$) 80.0% 80.0% 40.0%			

LG, low grade; HG, high grade.

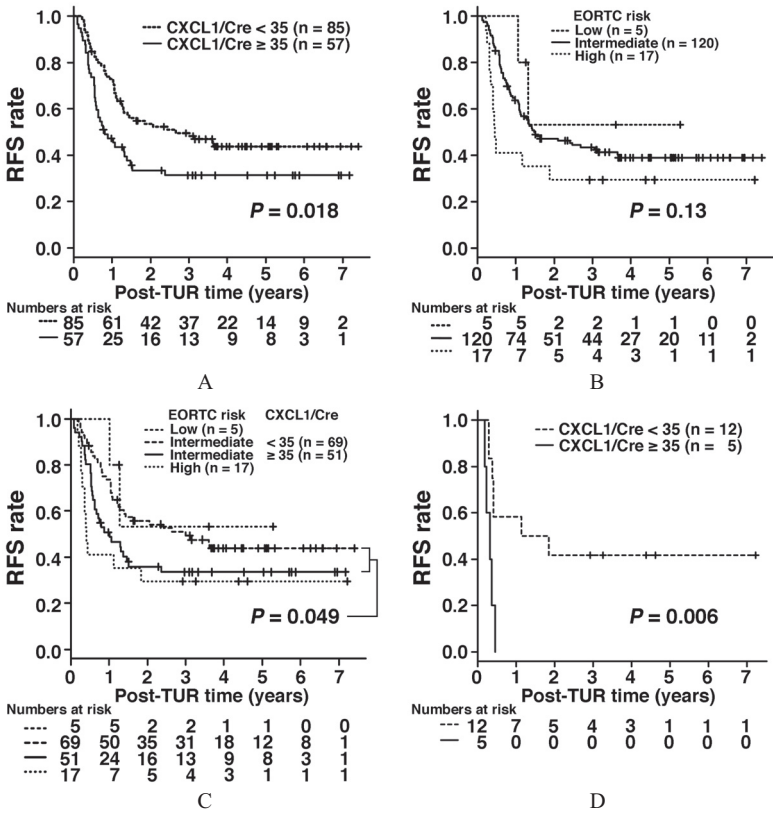

Fig. 3. Kaplan-Meier plots for post-TUR RFS. A: Patients stratified by CXCL1/Cre with a cutoff of 35 pg/mg. B: Patients stratified by EORTC risk score for post-TUR risk. C: Patients with EORTC intermediate-risk stratified by CXCL1/Cre with a cutoff of 35 pg/mg. D: Patients with EORTC high-risk stratified by CXCL1/Cre with a cutoff of 35 pg/mg.

significant correlations between high expression of CXCL1 and poor prognosis in several kinds of cancer, including ovary [12] and colorectal [13] cancers, as well as in BCa [8]. Although it is yet to be elucidated how clinically or biologically aggressive cancers tend to express high CXCL1, we speculate that it is associated with higher volume of tumor, stronger infiltrating trait, or activated interaction with inflammatory cells in the tumor microenvironment.

ROC analysis in the current study showed an AUC of 0.84 for CXCL1/Cre, suggesting that urine CXCL1 may be a useful detection marker for BCa. Other

Table 3
Univariate and multivariate analyses of time to post-TUR recurrence (Cox proportional hazards model with time)

	Univariate		Multivariate	
	P value	HR (95% CI)	*P* value	HR (95% CI)
Smoking	1.00	1.00 (0.64–1.58)	0.70	1.11 (0.69–1.74)
PH of UTUC*	0.75	1.12 (0.56–2.23)	0.93	0.96 (0.47–1.98)
Urine CXCL1/Cre ($\geqslant$ 35)	0.011	1.75 (1.13–2.69)	0.009	1.97 (1.19–3.28)
Urine cytology (4 or 5)	0.13	1.43 (0.90–2.27)	0.94	1.02 (0.60–1.72)
EORTC recurrence risk	0.14 (Wald test)		0.046 (Wald test)	
Low	(Ref.) —	—	(Ref.) —	—
Intermediate	0.46	1.71 (0.42–6.96)	0.58	1.50 (0.36–6.16)
High	0.15	2.98 (0.67–13.4)	0.13	3.24 (0.71–14.8)

HR, hazard ratio; PH, past history; UTUC, upper tract urothelial cancer.

commercially-available urine biomarkers such as BTA-trak and NMP22 were reported to have AUCs of 0.82 and 0.89, respectively [14,15]. Although it is not possible to compare results based on different patient populations, CXCL1/Cre appears to have a comparable overall diagnostic ability to detect BCa.

It should be noted that the sensitivity of CXCL1/Cre for detecting low-grade tumors was superior to that achieved by urine cytology. A meta-analysis by Lotan and Roehrborn [16] showed that urine cytology had a sensitivity of 20–50% and specificity of $>$ 80%. In contrast, the present study demonstrated a sensitivity of $>$ 50% for CXCL1/Cre for the detection of low-grade BCa. These results indicate that CXCL1/Cre may be useful for reducing the incidence of low-grade tumors that could be misdiagnosed on the basis of urine cytology test alone.

We also demonstrated the usefulness of urine CXCL1 for predicting intravesical recurrence of BCa after TUR. The EORTC risk stratification is one of the most commonly used risk criteria systems in this regard [5]. Patients with higher CXCL1/Cre values had poorer prognoses in each EORTC risk group. CXCL1/Cre was particularly successful in separating patients with intermediate-risk disease, which accounted for the majority of patients in the present study, consistent with a large-scale combined analysis reporting that intermediate-risk patients accounted for approximately 80% of those with non-muscle invasive BCa [7]. This group comprises a variety of diseases with a wide range of clinical aggressiveness, for whom the optimal treatment remains controversial [5,7]. A biomarker that could improve the outcome prediction in EORTC intermediate-risk patients would thus be a valuable addition. CXCL1/Cre could be such a complementary biomarker. Although EORTC intermediate-risk patients can be subclassified by the recurrence score (1–4 and 5–9) [7], there was still a significant difference in RFS between those with good- and poor-risk disease.

A potential weakness of the EORTC risk-stratification system is its reliance on pathological findings in TUR specimens. In this regard, an effective urine biomarker could provide important information for individualized treatment and follow-up prior to TUR. For instance, Colombo and colleagues reported the effectiveness of neoadjuvant intravesical mitomycin C [17], but the population most likely to benefit from preoperative instillation of mitomycin C remains unknown. Urine biomarkers including CXCL1/Cre have the apparent advantage of preoperative outcome prediction and may thus be useful for helping decision-making regarding the use of chemotherapy in the neoadjuvant setting.

The present study had several limitations. It was a case-control study, and post-TUR treatments such as intravesical Bacillus Calmette-Guerin instillation were not incorporated in the analysis. Additionally, since the present study is a transverse design, we did not examine urine samples longitudinally in the same patients during their follow-up periods after TUR. Another limitation was the possible occurrence of false-positive results caused by bacterial UTI; urine from patients with UTIs contains high levels of CXCL1 derived from leukocytes [18], but not from tumors [8], thus limiting the diagnostic ability of CXCL1 in patients with UTIs. However, we excluded urine from patients with obvious UTIs from the current analysis to minimize the effects of this factor. Further prospective studies are needed to provide an appropriate benchmark and to validate the results of this study in comparison with other biomarkers. The cutoff value of 35 pg/mg for the post-TUR recurrence prediction model has been determined as the value to separate the cohort with the best statistical significance based on Kaplan-Meier method, which should be also validated externally in the future study using another cohort.

In conclusion, we identified CXCL1/Cre as a promising urine biomarker for tumor detection and post-

TUR outcome prediction in patients with BCa. Our data indicated that urine CXCL1/Cre may be a useful complementary marker for BCa for use in combination with urine cytology tests and risk-stratification systems such as EORTC.

Conflict of interest

S.H. and K.K. are employees of Toray Industries Inc. The all remaining authors declare that there are no conflicts of interest.

Abbreviations

AUC,	area under the curve;
BCa,	bladder cancer;
CXCL1,	chemokine (C-X-C motif) ligand 1;
CXCL1/Cre,	urine CXCL1 concentration normalized by urine creatinine;
EORTC,	European Organisation for Research and Treatment of Cancer;
ROC,	receiver-operating characteristic;
TUR,	transurethral resection;
RFS,	recurrence-free survival

Supplementary data

The supplementary files are available to download from http://dx.doi.org/10.3233/CBM-150472.

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